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Second-Generation Bioethanol Production through a Simultaneous Saccharification-Fermentation Process Using *Kluyveromyces Marxianus* Thermotolerant Yeast

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Abstract

Due to the present renewable fuels demand increase, reduction of second-generation bioethanol production cost is pursued, since it is considered the most promising biofuel, but not yet economically viable. A proposed solution is its production through a simultaneous saccharification and fermentation process (SSF); however, it is necessary to apply temperatures above 40°C, which reduce the viability of traditional ethanologenic yeasts. As consequence, the use of thermotolerant ethanologenic yeast has been suggested, among which the yeast *Kluyveromyces marxianus* stands out. This chapter addresses the production of second-generation bioethanol through the SSF process, emphasizing the potential of *K. marxianus* to transform lignocellulosic biomass as agave bagasse. As result, it is proposed to direct the second-generation bioethanol production to the SSF process employing thermotolerant yeasts, to increase process productivity, and addressing the economic barriers.

Keywords: bioethanol, simultaneous saccharification and fermentation (SSF), thermotolerant yeasts, *Kluyveromyces marxianus*, agave bagasse

1. Introduction

The consumption of fossil fuels derived from petroleum is one of the main sources of pollution of the environment, in addition to its expensive and decreasing production, whereas its demand is increasing [1]. This is why countries around the world have directed their policies

toward the biofuels usage, which are sustainable, biodegradable, with high combustion efficiency, and their development generates manufacturing and investment jobs, promoting the de agricultural sector development, as well reducing greenhouse gases [2, 3]. This way, the use of biofuels such as bioethanol is pursued to reduce dependence on fossil fuels and contribute to meet the future demands of energy in the world, and at the same time meeting the carbon dioxide emissions reduction goals specified in the Kyoto Protocol [4]. Therefore, it is expected that by 2050 biofuels contribute 30% of the world's fuel demand [5].

Economically viable bioethanol production still has to date challenges to overcome. This chapter addresses the lignocellulosic biomass utilization for second-generation bioethanol production through a simultaneous saccharification and fermentation process, utilizing thermotolerant yeasts such as *K. marxianus*.

2. Bioethanol

Bioethanol is one of the most used biofuels with a worldwide production of around 27 billion gallons per year [2, 6]. This biofuel, defined as ethanol produced from biomass has characteristics such as low combustion temperature, high octane number, and lower evaporation loss compared to gasoline [7, 8]. Disadvantages of bioethanol compared to gasoline are its lower energy density and vapor pressure, as well as water miscibility and corrosive capacity [9].

Bioethanol can be mixed with gasoline in 10% (E10), 20% (E20), and 22% (E22) proportions, without the need to make mechanical modifications in combustion vehicles [9]. There are even current designs by some manufacturers that allow vehicles to use up to 85% ethanol [10] and in Brazil more than 20% of cars can use 100% ethanol as fuel [2]. The main purpose of bioethanol, when mixed with gasoline is as an oxygenating agent. Mixed with gasoline, ethanol provides advantages such as increased gas volume change, better combustion, and reduced carbon dioxide emission [11]. It has also been shown that bioethanol can significantly reduce SO₂ emissions when mixed with 95% gasoline. This is because the fuel added with bioethanol increases its oxygen content, causing a better oxidation of hydrocarbons and decreasing the emission of greenhouse gases [4].

The main bioethanol producing countries are currently the United States and Brazil, generating up to 70% of world production [12]. However, the bioethanol industry has expanded to other countries such as China, Argentina, and the European Union due to this product increased demand [13]. In the case of the United States, there has been a dramatic increase in bioethanol production from 175 million gallons in 1980 to 14,810 million gallons in 2015 [14].

2.1. First- and second-generation bioethanol

Bioethanol is currently obtained in commercial quantities mainly from the fermentation of simple sugars using food inputs such as corn, sugar cane, and sorghum as raw material. The bioethanol obtained from this class of substrates is called first generation bioethanol [15]. The viability of the production of first-generation biofuels is questionable, due to their associated

conflicts, such as ethical aspects and their high-cost since the raw materials are linked to the food market, which affects the final price of the product [16].

Given the problems of first-generation fuels, an alternative would be second-generation fuels, where fermentable sugars are derived from lignocellulosic biomass, which are present in agro-industrial wastes. By using industrial wastes as a raw material, pollution is reduced by the elimination of these potentially polluting wastes, as well the materials being of low-cost and its handling and conservation is efficient and economical [17]. Biofuel production such as second-generation bioethanol is considered one of the most promising strategies to replace non-renewable fossil fuels because it does not interfere with the materials available for human or animal consumption, at the same time as collaborating with sustainable development [18, 19].

As a disadvantage, a technological investment is necessary for the treatment of lignocellulosic biomass, and currently, its production is not economically sustainable [20, 21].

Currently, second-generation bioethanol is produced mainly in pilot plants and most commercial plants have been built in the last decade in Denmark, Finland, Spain and Italy, and the United States [22] and due to the challenges that its commercialization still represents the design and optimization of different processes for second-generation bioethanol production has been promoted to reduce production costs.

3. Process configuration for second-generation bioethanol production

There are different process configurations for bioethanol production, but all of them include the steps of raw material pretreatment to achieve biomass components solubilization and separation (cellulose, hemicellulose, and lignin); lignocellulosic material hydrolysis to degrade its components and obtain simple sugars; and the fermentation of the substrate to transform the sugars into bioethanol. Reported processes vary mainly in the number of stages and bioreactors needed, which present different pH conditions, oxygenation, sugar concentration, and temperature. **Figure 1** shows the main process configurations and their stages, while **Table 1** lists the main characteristics of each one of these configurations [23].

The CBP proposes cellulase enzyme production by microorganisms integrated into the fermentation, reducing the enzyme cost in the bioethanol production. However, currently, this process is in its early stages of development since a limited number of microorganisms capable of generating economically viable enzymes are reported. Furthermore, there is no microorganism or microorganism consortium that generates cost-effective bioethanol through CBP at the industry level [29]. In consideration to this situation, the development of genetically modified microorganisms able to produce these enzymes with an economically viable concentration and activity is one of the most promising options. Within the genetically modified microorganisms, yeasts have been one of the most used. Hasunuma and Kondo [30] presented a review of the development of yeast cells for second-generation bioethanol production through CBP. Within their study, they conclude that the combination of cell surface

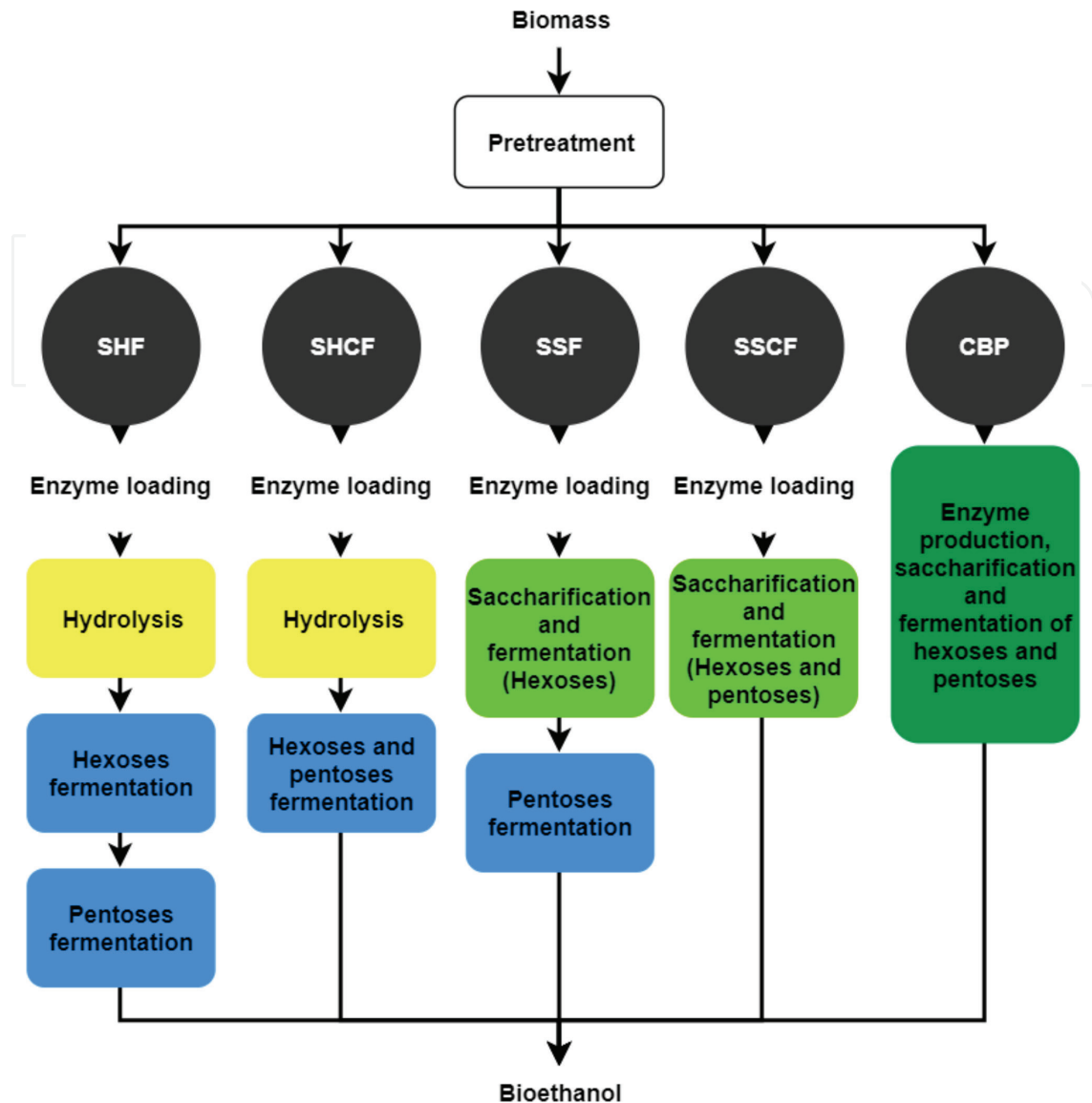


Figure 1. Processes for second-generation bioethanol production. Each box refers to a bioreactor. The pretreatment consists of a chemical hydrolysis. Sequential hydrolysis and fermentation (SHF); sequential hydrolysis and cofermentation (SHCF); simultaneous saccharification-fermentation (SSF); SSCF: Simultaneous saccharification-cofermentation (SSCF); consolidated bioprocess (CBP).

engineering and metabolome are an efficient proposal for the development of CBP yeasts strains. Favaro et al., Mattam et al., Liao et al., and Van et al. [31–34] review the possibilities of recombinant yeasts generation for second-generation bioethanol production through CBP.

In the rest of the mentioned processes in **Figure 1**, the cellulases are added beforehand SHF or during the fermentation process SSF. These enzymes can be purchased commercially through different companies responsible for selecting enzymes with the best characteristics to perform these processes. The current commercial cellulases are produced mainly by fungi, bacteria, and yeasts, although they can also be produced by plants and ruminants [35].

Process	Acronym	Main characteristics	Strain, substrate, temperature, % yield
Sequential hydrolysis and fermentation	SHF	The biomass hydrolysis and sugar fermentation are carried out sequentially in separate bioreactors. In this process the optimum temperatures for enzymatic hydrolysis and fermentation are different and it is necessary to cool the hydrolysate after the enzymatic hydrolysis to start fermentation. To achieve pentoses consumption it is necessary to add microorganisms capable of converting pentoses into ethanol at the end of hexoses fermentation. This is one of the most expensive processes, with long times, where enzymatic inhibition by the sugars generated can be present.	<i>Saccharomyces cerevisiae</i> 11.25% Bambú 30°C, 41% Sindhu <i>et al.</i> ²⁴
Sequential hydrolysis and cofermentation	SHCF	Unlike the SHF process, in this process the hydrolysis and fermentation of hexoses and pentoses is carried out in the same bioreactor. It is necessary the use of yeasts with the capacity to metabolize pentoses or the addition of a microbial consortium that together manage to assimilate both hexoses and pentoses in the medium.	<i>Scheffersomyces shehatae</i> 10% Bagazo caña 30°C, 82% Dussan <i>et al.</i> ²⁵
Simultaneous saccharification-fermentation	SSF	Combines the enzymatic hydrolysis and fermentation of hexoses in a single bioreactor simultaneously, increasing the efficiency of enzymatic hydrolysis by eliminating product inhibition, decreasing the risk of microbial contamination by being a single process and increasing bioethanol productivity compared with the SHF process. The pH and temperature values for these processes have been shown to be very rigid since the optimum pH and temperature values of the enzymes and yeasts used are compromised. At the end of the fermentation of hexoses, the addition of microorganisms capable of converting pentoses into ethanol is required.	<i>Kluyveromyces marxianus</i> 10% Pulpa de Zanahoria 42°C, 92% Yu <i>et al.</i> ²⁶
Simultaneous saccharification-cofermentation	SSCF	This process is similar to SSF with the difference that it uses yeasts capable of fermenting both hexoses and pentoses in the same bioreactor. This promotes the use of a greater amount of substrate, which can increase the production of ethanol and productivity increase by reducing the times of each stage.	<i>Saccharomyces cerevisiae</i> 10% Pícea 34°C, 85% Bertilsson <i>et al.</i> ²⁷ <i>Scheffersomyces shehatae</i> 10% Almidón 30°C, 29% Tanimura <i>et al.</i> ²⁸
Consolidated bioprocess	CBP	The enzyme production, enzymatic hydrolysis and fermentation of the sugars are integrated into the same bioreactor. As result, the addition of external cellulases is not necessary.	

Table 1. Main characteristics of the second-generation bioethanol production processes accompanied by an example of its implementation in the literature [23–28].

3.1. Sequential hydrolysis and fermentation (SHF)

This process is carried out in two stages, where hydrolysis and fermentation operate in different procedures. First, the enzymatic cocktail is used to hydrolyze the pretreated lignocellulosic biomass to obtain sugar monomers. The resulting hydrolyzate is subsequently used as a substrate for the fermentation process of sugars to ethanol [36]. The cellulose hydrolysis process through cellulases is the most feasible method for the liberation of sugars since in optimum conditions yields greater than 90% can be obtained. Chandel *et al.* [37] reviewed the techniques developed in molecular biology and cellulase engineering, as well as the application of cellulases for cellulose hydrolysis.

The main disadvantage of the SHF process is that both stages operate in their respective optimal conditions, thus more processing time is necessary. In addition, the hydrolytic enzymes employed can suffer from product inhibition. These characteristics impact on the productivity of the process [25]. The enzyme cost contributes significantly to the second-generation bioethanol final price, which is why more research is needed in order to reduce saccharification costs through the use of cellulases [36, 37]. In order to make second-generation bioethanol production affordable, cellulase cost must be decreased, and one solution is to increase its activity, which can be achieved through SSF processes at optimum temperatures of the enzymes employed [38].

3.2. Simultaneous saccharification-fermentation (SSF)

The main characteristic of the SSF process is that the stages of enzymatic hydrolysis and fermentation are carried out simultaneously. This reduces the energy investment, and therefore, the operating costs, in addition to optimizing the process by reducing the time needed for each of them. These stages favor a greater enzymatic activity of the cellulases eliminating product inhibition since the sugars are metabolized by the yeasts simultaneously as they are released in the hydrolysis. In general, through the SSF process, higher ethanol yields have been obtained compared to SHF, with increases from 13 to 30% [39]. The main disadvantage of the SSF process is the cellulase activity optimal temperature (45–60°C), which is higher than that required for the yeast growth and fermentation (30–35°C) [40, 41]. Besides, fermentation is an exothermic process, so as the fermentation progresses the temperature increases [42]. Therefore, the temperature is one of the main factors that must be considered when establishing a SSF system.

3.3. Effect of temperature in SSF process

Fermentation at high temperatures presents advantages such as bioreactor cooling costs reduction and ethanol extraction promotion, reducing its toxic effects on yeasts. Moreover, it is possible to do this process in warm climate countries [43]. In a study conducted by Abdel-Banat et al., an increase of 5°C in the fermentation process, a reduction of enzyme cost up to 50% was observed. However, high temperatures generate yeast growth inhibition, decrease in the cell cycle, increase in fluidity and reduction of the plasma membrane permeability, intracellular pH reduction, breakage of cytoskeleton filaments and microtubules, proteins synthesis repression, mutation frequency increment, and inefficient damaged DNA repair. All the above effects reduce the yeast viability and decrease the bioethanol production yield. Therefore, the use of thermotolerant yeasts in the SSF process for second-generation bioethanol production is proposed as a promising option [23].

4. Yeasts in SSF processes

Although traditionally *S. cerevisiae* yeasts have been the most used in fermentation processes, the production of second-generation bioethanol confronts these microorganisms to conditions not found in traditional fermentation processes [44, 45].

In a SSF process, the selected yeast must be a thermotolerant strain. Thermotolerant yeasts are those that have an optimal growth at temperatures equal to or greater than 40°C [46]. During the last years, potential industrial applications of thermotolerant yeasts have been developing, such as prebiotic and probiotic agents, biomass, and recombinant protein production, as well as bioethanol production [47]. Bioethanol production through SSF process using thermotolerant yeasts generates a reduction in investment costs, such as the industrial equipment needed, lower contamination degree, and decreased process time [17].

The most known non-*Saccharomyces* yeast species used in SSF processes are *K. marxianus*, although there are also reports of other species such as *K. fragilis*, *H. polymorpha*, and *P. pastoris* [47].

4.1. *Kluyveromyces marxianus*

K. marxianus strains are phenotypically very diverse due to the great variety of habitats in which they have been isolated, resulting in a great metabolic diversity [48].

In general, they are considered GRAS (Generally recognized as safe), they are the eukaryotic cells that have presented the highest growth rate [49], they have an efficient ethanol production capacity up to 45°C, with thermotolerance up to 52°C, besides the genomes of some strains have been described [50–56]. Recently, studies have been carried out on the optimization of the metabolic engineering pathways in these yeasts [57], and genetic engineering has been used to obtain strains capable of producing heterologous proteins or metabolites such as lactate and xylitol [58, 59].

One necessary characteristic in sustainable bioethanol production is the fermentation of different sugars [60], innate in most of *K. marxianus* yeasts. These yeasts can ferment xylose, xylitol, cellobiose, lactose, and arabinose, both in liquid and solid medium, considered a great advantage compared with *S. cerevisiae* [61].

Whereas strains of *S. cerevisiae* have been obtained by genetic engineering with pentose metabolism [62], these strains still present different problems that must be solved [63], besides that they are not thermotolerant. Nitiyon et al. [64] reported that the yeast *K. marxianus* BUNL-21 presents a xylose to ethanol efficient conversion capacity, as well as thermotolerance. López-Alvarez et al. [65] obtained higher ethanol yields with *K. marxianus* UMPe-1 yeast compared with *S. cerevisiae* Pan-1. Lyubomirov et al. [66] and Kuloyo et al. [67] compared the ethanol production at temperatures of 35 and 40°C by the strains *K. marxianus* UOFS Y-2791 and *S. cerevisiae* UOFS Y-0528, concluding *K. marxianus* presents potential as an alternative to *S. cerevisiae* for the bioethanol production, as well as other metabolites such as 2-phenyl ethanol.

Due to the aforementioned characteristics, *K. marxianus* have been considered as one of the yeast with the highest potential for the second-generation bioethanol production, and a viable alternative compared with *S. cerevisiae* [68, 30].

4.2. *K. marxianus* in the bioethanol production through SSF

As a thermotolerant yeast and due to its ability to use various sugars as a carbon source, *K. marxianus* yeasts have been used widely for second-generation bioethanol production through SSF and SSCF processes (Table 2).

Kádár et al. [80] compared the yield in the second-generation bioethanol production by *K. marxianus* and *S. cerevisiae* yeasts, in an SSF process at 40°C. Having found no significant differences in the ethanol production with respect to SHF processes, it is suggested to carry out the fermentation processes with *K. marxianus* thermotolerant yeasts at temperatures above 40°C. Tomás-Pejó et al. [79] performed SSF processes with a *K. marxianus* thermotolerant yeast CECT 10,875 at 50°C. By using a feed back process they increased ethanol production by 20%. Hyun-Woo et al. [75] carried out an SSF process with a temperature change from 45 to 35°C at 24 h of the process using the thermotolerant yeast *K. marxianus* CHY 1612. This change generated an increase of 12 g/L of ethanol, compared to a SSF process carried out at a constant temperature of 45°C. Yu-Sheng et al. [73] studied the bioethanol production using a *K. marxianus*

Strain	Temperature (°C)	Carbon source	Ethanol (g/L)	g EtOH/g substrate	Productivity (g/L/h)	Reference
K 213	42	Water hyacinth	7	0.14	0.31	Yan <i>et al.</i> , 2015
MTCC 1338	42	<i>Moringa oleifera</i> softwood	16-21	-	0.19	Shanmugam <i>et al.</i> , 2014
NRRL Y-6860	45	Rice straw	11	0.24	1.44	Cunha <i>et al.</i> , 2014
UFV-3, ATCC 8554 y CCT 4086	37,42	Sugar cane bagasse	13-22	0.17-0.29	1.62-2.75	Costa <i>et al.</i> , 2014
BCRC 21363	37-45	Sugar cane bagasse	22-24	0.22-0.24	0.30-0.33	Yu-Sheng <i>et al.</i> , 2013
K 21	42	Carrot pomace	18-37	0.18	0.75-0.88	Chi-Yang <i>et al.</i> , 2013
CECT 10875	42	Wheat straw	11	0.18	0.36	Moreno <i>et al.</i> , 2013
CHY 1612	35,45	Barley straw	22-34	0.13-0.21	0.30-0.47	Hyun-Woo <i>et al.</i> , 2011
IMB 3	45	Kanlow switchgrass	22-32	0.27	0.30-0.44	Pessani <i>et al.</i> , 2011
CECT 10875	42	Barley straw	19-29	0.19	0.66-0.77	García-Aparicio <i>et al.</i> , 2011
IMB 1, IMB 2, IMB 3, IMB 4, y IMB 5	45	Kanlow Switchgrass	16-21	0.13-0.17	0.22-0.29	Faga <i>et al.</i> , 2010
6556, 397 y 2762	37	Corn cob, soybean cake	2-5	0.04	0.10	Zhang <i>et al.</i> , 2010
CECT 10875	50	Wheat straw	22-36	0.22-0.25	0.50-1.18	Tomás-Pejó <i>et al.</i> , 2009
IMB 4	37-45	Kanlow switchgrass	12-16	0.16-.21	0.17-0.22	Suryawati <i>et al.</i> , 2008
Y 01070	40	Paper sludge	8-17	0.13-0.28	0.11-0.24	Kádár y Réczey, 2004
CECT 10875	42	<i>Eucalyptus globulus</i> , Sorghum and wheat straw	16-19	0.16-0.19	0.22-0.26	Ballesteros <i>et al.</i> , 2004
ITS4SC	42	Rice and wheat Straw, Sugar cane bagasse	18-23	0.18-0.23	0.30-0.38	Madhurinarra <i>et al.</i> 2015
CK8	43	Rice husk	15	0.16	0-16	Nachaiwieng <i>et al.</i> 2015
K21	40	Taro waste	49	0.29	2.23	Wu <i>et al.</i> 2016

Table 2. Second-generation bioethanol production using *K. marxianus* thermotolerant yeasts in SSF processes [26, 50, 68-84].

thermotolerant yeast, through the SSF process in a rotating reactor, which allowed a constant exchange of biomass that was in contact with the yeast, concluding that through this process bioethanol production has commercial potential. Wu et al. [84] implemented a SSF process with a high solid load of taro waste using *K. marxianus*, reaching 94% of theoretical yields in 20 h of fermentation, which was reflected in high process productivity (Wu et al. [84]).

With the previous reports, we observed that modifications to the SSF process using *K. marxianus* thermotolerant yeasts can increase ethanol production to economically viable levels.

5. Substrate selection for second-generation bioethanol production through SSF: agave bagasse case

Lignocellulosic biomass is a source of renewable energy, available in most of the world. However, its treatment is one of the main factors that increase the cost of second-generation bioethanol production. The biomass selection for this process is directly correlated with its availability in the production area, characteristics that depend on geographical variables [85]. In Mexico, agave bagasse is one of the most generated lignocellulosic materials, since it is an agro-industrial waste resulting from tequila and mezcal production. The blue agave (*Agave tequilana*) used for tequila production is cultivated mainly in the western region of Mexico. In general, the process of tequila production considers the use of blue agave plant cores, which are cooked in ovens or systems such as the diffuser. Afterward, they are pressed for their juice extraction, and the fructans present in the juice are then hydrolyzed to monosaccharides. The residue of this process is agave bagasse. It is estimated that 859,000 tons of agave are

processed per year to produce tequila, and approximately 343,600 tons of agave bagasse are generated. Agave bagasse can be used as livestock food, construction material, and for recycled paper elaboration [86], as well as a substrate for edible fungi growth [87]. However, most of it is incinerated, which generates large amounts of ash that can contaminate rivers, bodies of water and damage flora and fauna [88]. **Table 3** shows that agave bagasse has a higher cellulose proportion, compared to main lignocellulosic biomass used for bioethanol production.

Hernández-Salas et al. [90] obtained a sugar yield of 12–58% by hydrolysis of agave bagasse using an alkaline-enzymatic treatment, while under the same conditions with sugarcane bagasse the yield was lower, with values of 11–20% [90]. Therefore, according to its production and composition, agave bagasse can be considered a promising source of fermentable sugars for bioethanol production. **Table 4** shows studies for bioethanol production using agave bagasse.

Caspeta et al. [92] released 91% of agave bagasse sugars during saccharification and produced 64 g/L of ethanol after 9 h of fermentation with *S. cerevisiae* SuperStart yeast, this being the highest yield obtained with agave bagasse.

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Agave bagasse	42.0	20.0	15.0
Sugar cane bagasse	40.0	27.0	10.0
Corn stover	35.0	14.4	21.5
Corn cob	33.7	31.9	6.1
Wheat straw	32.9	24.0	8.9

Table 3. The lignocellulosic composition of agroindustrial wastes used in second-generation bioethanol production [89].

Yeast	Process	Ethanol (g/L)	Yield	Productivity ^a	Reference
<i>S. cerevisiae</i>	SHF	7	0.12	0.12	Hernández-Salas <i>et al.</i> , 2009
<i>Pichia caribbica</i> UM-5	SHF	18	0.18	0.15	Saucedo-Luna <i>et al.</i> , 2011
<i>S. cerevisiae</i> SuperStart	SHF	64	0.25	1.33	Caspeta <i>et al.</i> , 2014
<i>S. cerevisiae</i> Thermosacc	SHF	31	0.15	0.52	Montiel <i>et al.</i> , 2016
<i>S. cerevisiae</i> ATCC 4126	SHF	65	0.26	0.77	Rios González <i>et al.</i> 2017

Productivity is obtained considering total time of hydrolysis and fermentation.

Table 4. Bioethanol production from agave bagasse [90–94].

Rios González et al. [94] managed to implement a process of autohydrolysis pretreatment which allowed preserving the glycan content in agave bagasse, achieving a high digestibility in the hydrolysis process for its subsequent fermentation to ethanol with a strain of *S. cerevisiae*.

Through a simulation program analysis, Barrera et al. [95] carried out the technical and economic evaluation of bioethanol production, considering sugarcane bagasse, and agave bagasse as lignocellulosic biomass substrates. The results showed a lower production cost using agave bagasse (1.34 USD/gallon), compared to sugarcane bagasse (1.46 USD/gallon), suggesting that this result is due to the lower processing cost required for agave bagasse and its low lignin content [95].

Agave bagasse, besides being a good source of sugars for bioethanol production, is considered one of the best agro-industrial residues generated in the Mexico region, to be used in solid state fermentation processes [3], as well as for succinic acid production [96].

It is worth highlighting the scarce reports of bioethanol production from agave bagasse using non-*Saccharomyces* strains, as well as there are only reported SHF processes with this material, which represents a study opportunity to use this substrate in more efficient processes such as SSF.

6. Conclusion

Dependence on fossil fuels has led to a high degree of pollution on the planet, as well as low availability and an increase in its price, which forces the pursuit of new sources of energy. The use of second-generation bioethanol is a promising option to face this problem. However, currently, its production is not affordable, which has prevented its commercialization. Although metabolic engineering in conjunction with bioprocess optimization is recommended techniques for bioethanol cost-effective production, these are still in development, which contrasts with the widely used and perfected yeast selection techniques. These approaches can be used to find thermotolerant yeasts such as *K. marxianus* for their application in the second-generation bioethanol production through SSF processes to overcome the economic challenges in the production of this biofuel.

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